Variation in shoot tolerance mechanisms not related to ion toxicity in barley

Joanne Tilbrook^{1,2}, Rhiannon K. Schilling^{1,2}, Bettina Berger^{2,3}, Alexandre F. Garcia^{1,2,3},
Christine Trittermann^{1,2}, Stewart Coventry², Huwaida Rabie^{4,6}, Chris Brien⁴, Martin Nguyen⁴,
Mark Tester⁵, Stuart J. Roy^{1,2,*}

Affiliations

1. Australian Centre for Plant Functional Genomics, University of Adelaide, PMB1, Glen
Osmond, SA 5064, Australia

2. School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, SA
5064, Australia

3. The Plant Accelerator, Australian Plant Phenomics Facility, University of Adelaide, PMB1,
Glen Osmond, SA 5064, Australia

4. School of Information Technology and Mathematical Services, University of South
Australia, Mawson Lakes, SA 5095, Australia

5. King Abdullah University of Science and Technology (KAUST), Biological and
Environmental Sciences and Engineering (BESE), Thuwal 23955-6900, Saudi Arabia

6. Bethlehem University, Rue des Freres #9, Bethlehem, West Bank, Palestine

*Corresponding author

Stuart J. Roy, The University of Adelaide, PMB1, Glen Osmond, SA 5064, Australia.

stuart.roy@adelaide.edu.au

22 **Summary**

23 Salinity reduces the grain yield of cereal crops. In this study, non-destructive and destructive
24 phenotyping was used to evaluate 24 predominately Australian barley lines at 0, 150 and 250
25 mM NaCl. Lines with higher salinity tolerance were better able to maintain their growth rates
26 shortly after salt treatment and exclude Na⁺ from their shoots. This study shows that variation
27 in shoot tolerance mechanisms not related to ion toxicity exists in barley and suggests that
28 breeding new varieties with increased shoot ion independent tolerance is possible.

29 **Abstract**

30 Soil salinity can severely reduce crop growth and yield. Many studies have investigated salinity
31 tolerance mechanisms in cereals using phenotypes that are relatively easy to measure. The
32 majority of these studies measured the accumulation of shoot Na^+ and the effect this has on
33 plant growth. However, plant growth is reduced immediately after exposure to NaCl, before
34 Na^+ accumulates to toxic concentrations in the shoot. In this study, non-destructive and
35 destructive measurements are used to evaluate the responses of 24 predominately Australian
36 barley lines at 0, 150 and 250 mM NaCl. Considerable variation for shoot tolerance
37 mechanisms not related to ion toxicity (shoot ion independent tolerance) was found, with some
38 lines being able to maintain substantial growth rates under salt stress while others stop growing.
39 *Hordeum vulgare* L. spp *spontaneum* accessions and barley landraces predominantly had the
40 best shoot ion independent tolerance, although two commercial cultivars, Fathom and Skiff,
41 also had high tolerance. The tolerance of cv Fathom may be due to a recent introgression from
42 a *Hordeum vulgare* L. spp *spontaneum*. This study shows that the most salt tolerant barley lines
43 are those that contain both shoot ion independent tolerance and the ability to exclude Na^+ from
44 the shoot (and thus maintain high K^+/Na^+ ratios).

45

46 **Keywords:**

47 *Hordeum* spp., osmotic stress, plant growth, plant phenomics, salt tolerance

Introduction

A substantial increase in food production is required to meet the demands of a growing and wealthier world population. Advances in breeding and agronomic practices have achieved, on average, a linear increase in cereal production of 32 million metric tons per year over the past 50 years (Tester and Langridge 2010). However, this current increase in production is insufficient to meet future demands as an increase in 44 million metric tons per year is required between now and 2050 (Tester and Langridge 2010). Achieving this target will be challenging due to increasing fertiliser costs, reduced water availability and quality, variable climates and loss of farmland caused by increasing urbanisation, desertification and salinization (Cassman *et al.* 2003).

Soil salinity severely reduces crop growth and yield (Munns and Tester 2008; Munns *et al.* 2012). Within minutes of exposure to salt, cell expansion, leaf expansion, photosynthesis, transpiration and tillering are reduced (Yeo *et al.* 1991; Passioura and Munns 2000; Fricke and Peters 2002; Colmer *et al.* 2005; Eynard *et al.* 2005; Munns and Tester 2008; Rahnama *et al.* 2011). These effects take place before ions, such as sodium (Na^+) and chloride (Cl^-) accumulate in the shoot and has thus been termed osmotic stress (Munns and Tester 2008) or shoot ion independent stress (Roy *et al.* 2014). When salts accumulate to toxic concentrations in the shoot, especially in older leaves, a secondary inhibition of growth occurs through damage to plant metabolism and ion imbalances (Wyn Jones *et al.* 1979; Bhandal *et al.* 1988; Munns *et al.* 1995; Blaha *et al.* 2000; Munns 2002; Munns and Tester 2008; Jacoby *et al.* 2016). These effects occur weeks to months following salt application and are termed ionic stress or shoot ion dependent stress (Munns *et al.* 1995; Munns and Tester 2008; Roy *et al.* 2014).

Plants have evolved numerous mechanisms to detect and respond to the effects of salt stress including a range of signal transduction mechanisms (Kudla *et al.* 2010; Choi *et al.* 2014; Schmöckel *et al.* 2015; Thoday-Kennedy *et al.* 2015), Na^+ and Cl^- exclusion from the shoot (Munns *et al.* 2006; Munns and Tester 2008; Teakle and Tyerman 2010), accumulation of Na^+ and Cl^- in vacuoles (Flowers and Colmer 2008; Munns and Tester 2008; Shabala 2013), synthesis of compatible solutes (Munns and Tester 2008; Shabala 2013), reduced K^+ efflux from the root (Chen *et al.* 2005; Chen *et al.* 2007; Chen *et al.* 2008) and maintenance of plant growth (Munns and Tester 2008; Rajendran *et al.* 2009; Roy *et al.* 2014).

81 Most studies investigating salinity tolerance mechanisms in cereals use phenotypes that are
82 easily measured: Na⁺ exclusion from the shoot and maintenance of high K⁺/Na⁺ ratios by
83 elemental analysis; synthesis of compatible solutes for tissue tolerance by metabolomics
84 analyses, efflux of K⁺ from the root by microelectrode ion flux estimation (MIFE); overall
85 salinity tolerance by measuring plant biomass or, occasionally, grain yield (Zhu *et al.* 2001;
86 Poustini and Siosemardeh 2004; Colmer *et al.* 2006; Chen *et al.* 2008; Cuin *et al.* 2008; Munns
87 and Tester 2008; Cuin *et al.* 2009; Widodo *et al.* 2009; Chen and Murata 2011; James *et al.*
88 2012; Munns *et al.* 2012; Shabala 2013; Adem *et al.* 2014). However, little is known about the
89 mechanisms contributing to shoot ion independent tolerance, which involves the maintenance
90 of plant growth during salt stress. Plant growth is severely reduced after exposure to salt, before
91 ions accumulate to high concentrations in the shoot and affect the processes in the shoot
92 (Munns *et al.* 1995; Munns and Tester 2008; Roy *et al.* 2014). It has been proposed that shoot
93 ion independent tolerance involves long-distance signalling mechanisms, which help maintain
94 plant growth (Roy *et al.*, 2014). However, investigating the maintenance of growth under salt
95 stress has been limited by the lack of techniques which allow non-destructive measurements of
96 plant growth through time.

97

98 In recent years non-destructive imaging and analysis has been successfully used to measure
99 plant growth rates under abiotic stresses, using either Red Green Blue (RGB) imaging or
100 infrared thermography (Sirault *et al.* 2009; Berger *et al.* 2010; Hairmansis *et al.* 2014; Honsdorf
101 *et al.* 2014; Schilling *et al.* 2014; Neilson *et al.* 2015; Parent *et al.* 2015, Al-Tamimi *et al.*
102 2016). Growth determined from digital images of shoot area allows for multiple measurements
103 of shoot biomass through time and is an ideal technology to determine the early shoot ion
104 independent effects of salinity on plant growth. The resources and technologies now exist to
105 phenotype many genotypes and identify those with high shoot ion independent and shoot ion
106 dependent tolerance in greenhouse conditions.

107

108 Barley (*Hordeum vulgare* L. ssp *vulgare*) is one of the more salt tolerant crops, able to grow in
109 higher concentrations of salt than wheat, rice or maize (Maas and Hoffman 1977; Colmer *et al.*
110 2005; Tilbrook and Roy 2014). However, growth of barley is still significantly affected by
111 salinity (Colmer *et al.* 2005). In Europe the highest barley yields are approximately 6-7 t/ha in
112 a favorable year (FAOSTAT 2015), while in Australia yields average 2 t/ha (ABARES 2014;
113 FAOSTAT 2015). One major abiotic stress contributing to this reduction in yield is soil salinity

(Rengasamy and Marchuk 2011). A better understanding of the genetic variation for salinity tolerance mechanisms within barley cultivars is required for future breeding improvement.

The work described here details the response of 24 lines of barley to salinity stress. Using non-destructive imaging, shoot growth rates were analysed to determine the shoot ion independent tolerance of each line. A large variation in shoot ion independent tolerance was observed between lines, particularly at concentrations of 250 mM NaCl. The lines that combined shoot ion independent tolerance with other tolerance mechanisms, such as ion exclusion, maintained relatively high shoot biomass over a longer time under 250 mM NaCl, and were typically *H. spontaneum*, landraces or cultivars bred recently using landraces.

Materials and Methods

Plant material

Twenty-four commercial and landrace barley lines (*Hordeum vulgare* L. ssp *vulgare* and *H. vulgare* L. ssp *spontaneum*) were selected for this study. These consisted of ten Australian malting varieties, eight Australian feed varieties, two Australian breeding lines (WI4304 (Obsa *et al.* 2016) and WI4330 (Ismagul *et al.* 2014), Barley Breeding Program, University of Adelaide), a Chinese feed variety, YU6472 (from China through the Tasmanian Institute of Agriculture and Grains Research & Development Corporation collaboration), which grows in coastal areas with saline soils (Tajbakhsh *et al.* 2006)), and three more diverse barley lines known to be tolerant to abiotic stresses: Sahara 3771 (selection from an Algerian landrace), Parent 19 (a Syrian breeding line obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA)) and CPI 71284-48 (*H. spontaneum*), derived from Commonwealth Plant Introduction # 71284) (Eglinton *et al.* 2004).

Experimental design

Plants were grown in a greenhouse and an automated phenotyping Smarthouse of the Australian Plant Phenomics Facility, The Plant Accelerator® (TPA) (Waite Campus, University of Adelaide, South Australia, Australia; longitude: 138.639933°, latitude: -34.971353°) between March and April (autumn) of 2012. A split plot experimental design was used, in which 24 varieties were assigned to plots that consisted of three consecutive pots within the same lane in the Smarthouse, with each pot containing a single plant of that line. The three treatment levels (0, 150 and 250 mM NaCl) were randomized within each plot. The design used to assign the varieties to main plots was a resolved, spatial design with rows and columns.

As the design is resolved, all 72 combinations of the lines and treatments occurred once in a replicate, with each of the eight replicates consisting of six lanes and 12 positions. The design was generated using DiGger (Coombes 2009), a package for the R statistical computing environment (The R Project for Statistical Computing, 2014).

Plant growth

Evenly sized seeds of each line were selected, placed into 50 mL polypropylene tubes and imbibed in reverse osmosis (RO) water at room temperature for 2 h. The tubes were drained of water, sealed and placed in the dark at 4°C for 3 days. CPI 71284-48 seeds were similarly imbibed but held for 1 extra day in the dark at 4°C to ensure uniform germination with the other lines used in the study. Free draining pots with a diameter of 145 cm and height of 190 cm were filled with a soil consisting of 50% (v/v) University of California mix, 35% (v/v) peat mix and 15% (v/v) clay loam. Four seeds from each line were sown 2 cm deep and 2 cm apart in each replicate pot (n = 7-8) and placed in the greenhouse. At sowing, the soil had a gravimetric water content close to 33% (w/w). To prevent excessive drying of the soil surface during seed germination, the pots were very lightly sprinkled with RO water each day. At the emergence of the 2nd leaf, the four plants were thinned to one evenly sized plant per pot. The plants were grown under natural light in a temperature-controlled greenhouse with a 22°C daytime temperature and 15°C at night.

Growth assay under control and salt stress conditions

When the majority of the seedlings had the 3rd leaf emerging, all pots were transferred into a Smarthouse of TPA and grown under natural light with a daytime temperature of 22°C and 15°C at night. CPI 71284-48 plants were slower to germinate and were at second leaf emerging when transferred to the Smarthouse. A deep saucer (160 cm × 160 cm × 90 cm) was placed under each pot and each pot with saucer was placed in an individual cart on the automated conveyor system, where they were imaged, weighed and watered to weight (25% w/w) over the following 21 days (Berger *et al.* 2012; Brien *et al.* 2013).

At the emergence of the 4th leaf for all plants except the 3rd leaf for CPI 71284-48, salt treatment was applied directly into the saucer in a 200 mL volume (Berger *et al.* 2012), to be taken up into the soil over the subsequent 2 h and 200 mL of RO water was used for the 0 mM treatment. Immediately after treatment, the pots contained 0, 110 or 180 mM NaCl, with the soil water content at 35% (w/w). Pots were then not watered until the soil dried to the target soil water

content of 25% (w/w). During this time, the NaCl concentration in the aqueous phase of the pots increased to 0, 150 (moderate salt stress) and 250 (high salt stress) mM, respectively, over a period of 4-6 days after treatment as the soil water content reduced due to evaporation and transpiration. Watering recommenced when the soil water content reached 25% (w/w) and was maintained daily to ensure pots were kept at this target water content.

Plant imaging

Images of plants were taken using a LemnaTec 3D Scanalyzer system (LemnaTec GmbH, Wuerselen, Germany). Three 2056 × 2454 pixels RGB images (one top view image and two side view images with a 90° angle to each other) were taken of each plant daily. Background-foreground separation was used to separate the plant from the background and erosion and dilation steps were used to remove noise from the images so that shoot area was not over-estimated (Berger *et al.* 2012; Hairmansis *et al.* 2014). The sum of plant pixels from three images was calculated and is referred to as the projected shoot area. The projected shoot area has been shown to be linearly correlated to shoot biomass in barley for plant sizes used in the present study (Rajendran *et al.* 2009; Golzarian *et al.* 2011; Hairmansis *et al.* 2014; Honsdorf *et al.* 2014). Plant health was determined by dividing the number of green pixels in each image by the number of total pixels that made up the image of the plant.

Leaf tissue sampling and analysis

Leaf blades, which were emerging at the time of treatment, were harvested 17 days after treatment, when they were fully expanded. Fresh weight and dry weight of the leaf tissue was recorded. Dry leaf tissue was digested in 10 mL of 1% HNO₃ (v/v) in a HotBlock (Environmental Express, Mount Pleasant, SC, USA) at 85°C for 4 h and leaf Na⁺ and K⁺ content measured using a flame photometer (Model 420, Sherwood Scientific, Cambridge, UK) (Shavrukov *et al.* 2010b).

Plant growth curve fitting

Indicators of plant growth were computed for each day of imaging as follows. A cubic smoothing spline (Shipley and Hunt 1996) was fitted to the projected shoot areas (kpixels) for each plant using the function “smooth.splines”, available in the standard R statistical computing environment (R Development Core Team, 2014). The parameter df of the function was set to 5, as this was judged to produce a fitted curve that followed the general growth trend

of the plants in this study. Absolute growth rates of shoots (AGR kpixels/day) were calculated for each plant for each day on which it was imaged as $AGR = dA/dt$, where A is the projected shoot area determined from the images. Similarly, relative growth rates (RGR kpixels/day/kpixel) were calculated for each plant for each day on which it was imaged as $RGR = (1/A)(dA/dt)$. The AGR was calculated as the first derivative of the fitted spline using the “predict.smooth.splines” function. The ratios of the relative growth rates were calculated for each triple of consecutive plants for each day on which they were imaged as $RGR_{salt} / RGR_{control}$ for each salt level. Average relative growth rates between day 0 and day 5 inclusive after salt treatment (a total of 6 days) were calculated for each plant as the geometric mean of their daily RGR values in this interval. The ion independent salt tolerance for this interval was obtained for each triple of consecutive plants as the ratio of the interval RGR for one salt level to the interval RGR for the control. Mixed model analyses were performed using areml-R (Butler *et al.* 2010), a package for the R statistical computing environment (The R Project for Statistical Computing, 2014).

Statistical analysis

Data in Supplementary Figure 1 was statistically analysed using a one-way ANOVA in GraphPad PRISM® v7.00 and Tukey’s test was used to determine means significantly different at a probability level of $P \leq 0.05$. A Pearson’s correlation analysis was performed in GraphPad PRISM® to determine the significance of correlations at a probability level of $P \leq 0.05$.

Results

Salt stressed barley has variable growth responses shortly after salt treatment

At the end of the experiment, 17 days after the salt treatment, significant differences in the size of different barley lines was observed in all three treatments (0, 150 and 250 mM NaCl) (Figure 1). Two of the lines with diverse genetic backgrounds, CPI 71284-48 and Sahara 3771, had a larger shoot biomass (projected shoot area) than the Australian cultivars in all treatments (Figure 1a, b, c). As expected, large reductions in projected shoot area were observed for all lines with increasing NaCl concentrations (Figure 1). Salinity tolerance was defined as the ability of a line to maintain shoot biomass under salt stress relative to control conditions after 17 days of treatment, calculated as (projected shoot area in salt)/(projected shoot area in control). Lines with salt tolerance ratios closer to 1 are better able to maintain their growth in salt stress conditions to levels similar to that in non-stressed conditions, and are therefore considered to be salt tolerant. Under moderate salt stress (150 mM NaCl), all lines were able

to maintain moderate biomass weights, with salt tolerance ratios between 0.3 and 0.7 (Figure 1d). Severe salt stress (250 mM NaCl) resulted in a much larger separation of the most salt tolerant lines (CPI 71284-48, Sahara 3771, Fathom, Parent 19 and Skiff) from those with poor salt tolerance (Gairdner, Navigator, WI4330 and Yarra) (Figure 1e).

Variation in shoot ion independent stress tolerance exists in barley

Non-destructive imaging of each line for 17 days after salt treatment enabled identification of varietal differences in shoot growth rates (projected shoot area). For all lines, it can be observed that increases in projected shoot area are severely slowed in plants grown in either 150 mM or 250 mM NaCl (Supplementary Figure 2). However, there are clear differences between different lines. CPI 71284-48 and Sahara 3771 continue to increase projected shoot area after exposure to 250 mM, while lines such as Gairdner and Navigator showed little increase in projected shoot area after exposure to 250 mM NaCl (Supplementary Figure 2).

In the control treatment (0 mM NaCl), all lines maintained a fairly steady relative growth rate of approximately 0.1 over the 19 days of observations (Figure 2a). After exposure to salt, the RGR of all individuals was either moderately reduced (150 mM NaCl) (Figure 2b), or severely reduced (250 mM NaCl) (Figure 2c). In both cases, shoot relative growth rates never recovered to the rates observed under non-stressed conditions.

The ability to maintain shoot growth under salt stress at rates similar to those observed in control conditions varied between lines (Figure 3). While most lines were able to maintain similar growth rates to control-grown plants when treated with 150 mM NaCl (having ratios between 0.8 and 1.0; relative growth rate at 150 mM/relative growth rate at 0 mM), a large variation in tolerance between the lines was observed at 250 mM NaCl. Three distinct groups of lines with different ratios of relative growth rates over the whole stress period are observed in Figure 3:

- Group 1: Lines that are able to maintain relative growth rates when exposed to 250 mM NaCl that are close to those measured in plants grown in 0 mM NaCl. Examples include CPI71284-48 and Sahara 3771. These appear to have both high ion independent and dependent tolerance
- Group 2: Lines that have a severe reduction in relative growth rate during the whole period the plants are exposed to 250 mM NaCl. Examples include Gairdner, Clipper and Keel. These appear to have low ion-independent tolerance and may or may not have

good ion tolerance. This cannot easily be resolved, as growth has been so significantly reduced by the rapid, ion-independent effects.

- Group 3: Lines that exhibit a severe reduction in relative growth rates, but then recover slightly approximately 10 days after addition of 250 mM NaCl, although with still much lower growth rates than seen in 0 mM NaCl. Examples include Baudin, Mundah and Barque 73. These lines may require a period of adjustment before activating both ion-independent and dependent tolerance

Shoot ion independent stress reduces the growth rate of barley immediately after the addition of NaCl. Over time, Na^+ and Cl^- accumulate in leaf tissue resulting in shoot ion dependent stress and further growth reduction. Therefore to assess the shoot ion independent stress tolerance on its own, before the compounding factors of ion accumulation, the difference between salt and control relative growth rates over the first 5 days after exposure to salt was used to calculate an average shoot ion independent tolerance for each line (Figure 4). Under 150 mM NaCl stress, most of the barley lines had good shoot ion independent tolerance (0.8 to 1.0), with Fathom and CM72 being able to maintain relative growth rates at 150 mM NaCl similar to that when grown in 0 mM NaCl (Figure 4a).

For plants grown in 250 mM NaCl, a large variation in the shoot ion independent tolerance was observed (Figure 4b). Fathom and Parent 19 were the best performing lines, with similar relative growth rates over the first 5 days after exposure to 250 mM NaCl compared to 0 mM NaCl (Figure 4b). WI4304 and Navigator had the lowest shoot ion independent tolerance (0.376 and 0.388, respectively) (Figure 4b). The remaining lines, from Clipper to CPI 71284-48 exhibited a range of shoot ion independent tolerance from 0.46 to 0.82 (Figure 4b). At 150 mM, a slight positive relationship between shoot ion independent tolerance (maintenance of growth over the first 5 days) and overall plant salinity tolerance (as determined at final imaging point) was observed (Figure 4c). At a high salinity treatment of 250 mM, there is a clear correlation between shoot ion independent tolerance and overall plant salinity tolerance (Figure 4d).

Na^+ exclusion from barley leaves only linked to salinity tolerance under high salinity stress

Under non-stressed and 150 mM NaCl conditions, there was little variation in leaf $[\text{Na}^+]$ and $[\text{K}^+]$ between lines (Figure 5), no relationship between leaf $[\text{Na}^+]$ and final projected shoot area 17 days after treatment and a negative relationship between leaf $[\text{Na}^+]$ and salinity tolerance

(Figure 6a, b). At 250 mM NaCl, several lines (CPI 71284-48, Sahara 3771, Mundah, Fathom, Skiff and Parent 19) accumulated low (<250 mM Na^+) concentrations of leaf $[\text{Na}^+]$, similar to the concentrations they accumulated in 150 mM NaCl (Figure 5 c, e). While many lines were able to maintain leaf $[\text{K}^+]$ levels similar to those in 0 mM NaCl (Figure 5 b, f), others, such as Keel, Hindmarsh, WI4304 and Clipper exhibited a doubling in leaf K^+ concentration and lower leaf water contents (Table 1). Many lines, however, accumulated very high $[\text{Na}^+]$ (>850 mM NaCl) in their leaves; these included Navigator, WI4330, WI4304, Hindmarsh and Gairdner (Figure 5e). In the 250 mM NaCl treatment there was a clear negative relationship between leaf $[\text{Na}^+]$ and both final projected shoot area (plant biomass) and salinity tolerance (Figure 6c, d). When grown in 250 mM NaCl a large variation in leaf health, the proportion of green shoot to total leaf pixel area, was observed between cultivars (Figure 6e) with lines that accumulated high leaf $[\text{Na}^+]$ exhibiting classic symptoms of salinity stress, including high levels of leaf senescence and thus reduced proportions of healthy green shoot area (Figure 6f).

Salinity tolerance in barley is a combination of many traits

When barley lines were ranked on their salinity tolerance at 250 mM NaCl (Table 1) and 150 mM NaCl (Supplementary Table 1) a number of observations can be made. In 250 mM NaCl, the barley lines with the highest salt tolerance are also those with the highest shoot ion independent tolerance, the lowest leaf Na^+ accumulation, the highest K^+/Na^+ ratios and the highest leaf health (Table 1). Consistently, those lines with the lowest salt tolerance also had the lowest shoot ion independent tolerance, highest leaf Na^+ accumulation, were unable to maintain leaf K^+/Na^+ ratios and had the lowest leaf health (or greenness) (Table 1). When ranked by salt tolerance, shoot ion independent tolerance or Na^+ exclusion, malting barley cultivars tended to have the poorest tolerance, while feed barley had moderate tolerance (Table 1; Supplementary Figure 1). Diverse genotypes had the best salt tolerance, with two exceptions being the malting barley Skiff and the feed barley Fathom which also had high salt tolerance. At 150 mM NaCl, plants which had good shoot ion independent tolerance and Na^+ exclusion tended to also have the greatest salinity tolerance, however, this was not as apparent as the pattern observed at 250 mM NaCl.

Discussion

In this study, non-destructive and destructive measurements are used to evaluate the responses of 24 barley lines under different salt concentrations. Considerable variation was shown to exist in barley for:

- Shoot ion independent tolerance, the response of plants that occurs before salt has had a chance to both accumulate in the shoot and affect processes in the shoot.
- Salinity tolerance, as quantified by projected shoot area after 17 days in salinity relative to the projected shoot area of plants grown in optimal conditions; and
- Na^+ and K^+ accumulation in leaf blades

It was found that the most salt tolerant barley lines are those that possess multiple salt tolerant mechanisms. Wilder accessions of barley had the greatest salt tolerance and had both high Na^+ exclusion and shoot ion independent tolerance. Interestingly, Skiff (a malting variety now grown as feed) and Fathom (a feed variety) had disproportionately higher salt tolerance compared to other cultivars of their type.

Variation for shoot ion independent tolerance in barley

An immediate reduction in plant growth can be observed for every line after the addition of NaCl (Figure 3 and Supplementary Figure 2), as determined by a reduction in the relative growth rates (Figure 3) of the plants and their shoot ion independent tolerance (Figure 4). Lines have been identified that are better able to maintain their growth when exposed to salt, such as Sahara 3771, Fathom, CPI-71284-48 and Parent 19 (Figures 3 and 4). While their growth rates slowed upon the application of salt, these lines were still able to expand their leaves and maintain shoot growth. This study also found that the growth of most lines was reduced under high salt for a prolonged period – the rapid reduction in growth was not reversed (Figure 3). Lines such as YU6472, Navigator, Commander and Keel, stopped growing when exposed to 250 mM NaCl. It is hypothesised that these lines may have closed their stomata, thereby limiting photosynthesis and consequently leaf expansion and tillering (Munns and Tester 2008). In addition to identifying lines that either maintained growth after salt stress or had their growth inhibited by salt, this study also identified a small number of lines that had an immediate reduction in growth rate but then showed growth rate recovery approximately 10 days after exposure to salt (Figure 3). This may be an adaptive response to the salinity treatment, where plant growth is delayed while necessary salt tolerance mechanisms are activated. Alternatively, it may be an escape mechanism, where plants temporarily suspend growth on exposure to high salt levels, thereby temporarily reducing damage, but then resume growth despite salt levels remaining high.

Mechanisms behind growth maintenance under salt still need to be elucidated

Mechanisms for shoot growth maintenance shortly after exposure to salinity before ions accumulate to high concentrations in shoot tissue (shoot ion independent tolerance) are as yet unknown (Roy *et al.* 2014). It seems likely to involve rapid long distance signalling components which occur immediately after exposure to salt, along with stomatal closure and synthesis of compatible solutes (Fricke *et al.* 2004; James *et al.* 2008; Sirault *et al.* 2009; Kudla *et al.* 2010; Mittler *et al.* 2011; Roy *et al.* 2014). Hydraulic signalling as well as non-hydraulic processes (such as transient calcium, reactive oxygen species and hormone fluxes, lasting minutes to hours), have all been implicated in the signalling of salt stress (Munns *et al.* 2000; Kudla *et al.* 2010; Mittler *et al.* 2011; Choi *et al.* 2014; Huber and Bauerle 2016). However, as can be observed from Figures 3 and 4 and Supplementary Figure 2, the reduction in growth rates of many lines exposed to salinity stress is maintained over a period of days, for much longer than the transient signals have been observed. It is thus likely that longer lasting responses (and possibly also signals) exist, in addition to the transient signals, which are responsible for maintaining slowed growth rates under salinity. Now that tolerant lines have been identified, the mechanisms behind their ability to maintain growth can be investigated. Forward genetic approaches, such as bi-parental quantitative trait locus (QTL) mapping or genome wide association mapping, are required to identify genetic loci linked to maintenance of growth under salt.

Performance in unstressed conditions does not predict good shoot ion independent tolerance

No relationship between the shoot ion independent tolerance and the initial projected shoot area (prior to salt treatment) or their relative growth rates under 0 mM NaCl treatment was observed for the barley lines grown in this study (Supplementary Figure 3). This suggests that the performance of barley lines under non-stressed conditions does not predict their performance under salt - e.g. there is no evidence that barley lines with the highest ion independent tolerance are the slowest growing. This is promising for plant breeders, as it will be possible to introduce mechanisms to maintain growth under salinity without adversely affecting the growth rate of plants in non-stressed environments. The finding that Fathom, a high yielding cultivar in the field (Paynter *et al.* 2014; Matthews *et al.* 2015; Victoria 2015; Wheeler 2015), has a high shoot ion independent tolerance suggests it is possible to introgress salinity tolerance traits into barley without detrimental effects on grain yield.

Multiple tolerance mechanisms are necessary for overall salt tolerance

In the quest to increase crop salinity tolerance, research is often focused on improving one mechanism of tolerance, be it exclusion of Na^+ from the shoot, maintenance of high K^+/Na^+ ratios in the shoot, tolerance of high Na^+ in the shoot, synthesis of compatible solutes or reducing efflux of K^+ from the root. While these traits are individually important, and manipulation of them in isolation has improved yield in the field (James *et al.* 2012), it is likely that a combination of these tolerance mechanisms will have the greatest impact on improving crop performance in a saline field. The introgression of a gene important in regulating shoot Na^+ exclusion, *TmHKT1;5-A*, from *T. monococcum* into the durum wheat (*T. durum* cv Tamaroi), resulted in improved yield in the field, but only in highly saline soils (James *et al.* 2012; Munns *et al.* 2012). The yield of the *TmHKT1;5-A* Tamaroi was similar to the Tamaroi parent under low and moderate salinity, suggesting that other traits, such as shoot ion independent tolerance, need to be introgressed into those lines to improve yield in mild and moderate saline environments.

Of the 24 barley lines in this study, it was those with both shoot ion dependent and shoot ion independent tolerance that were the most salt tolerant (Table 1). There was no evidence that a single tolerance mechanism dramatically improved salinity tolerance alone, similar to observations in wheat (Rajendran *et al.* 2009). The focus for pre-breeders and breeders in the future should be on the identification of the best traits and mechanisms to pyramid together in elite cultivars to obtain the most effective increases in salt tolerance that benefits crops growing under variable levels of salinity.

Introduction of wild barley tolerance mechanisms into commercial varieties is possible

The barley lines with the highest overall salinity tolerance were generally wilder accessions (Table 1), which are lower yielding than commercial cultivars (in terms of yield per plant) but are better adapted to more extreme environments. CPI 71284-48 is a *H. vulgare* spp *spontaneum* accession (Shavrukov *et al.* 2010a), which is found in environments with extreme stresses, such as high temperatures, drought and soil salinity; Sahara 3771 is a line derived from an Algerian landrace (Sutton *et al.* 2007) while Parent 19 is an ICARDA breeding line from Syria. Of the 24 barley lines used in this study, the majority of Australian cultivars bred for malting had lower salinity tolerance than those bred for feed (Table 1). The reason that these malting barleys generally have lower salinity tolerance than the feed barleys remains unclear. Feed barley often share similar pedigrees and are selected under the same field conditions as malting barley cultivars. It is unlikely that the lower salt tolerance in malting

barley is due to salinity tolerance mechanisms being incompatible with mechanisms for producing grain with high malting quality, as the malting cultivar Skiff has the best salinity tolerance of all cultivars measured (Table 1).

Interestingly, Skiff (malting barley) and Fathom (feed barley) have overall very high salinity tolerance, disproportionately so to others of their type. Fathom has CPI-71284-48 in its pedigree, while Skiff has an Algerian *H. vulgare* landrace, CPI-18197-48, in its background. It is likely that introgression of DNA from these two wilder accessions is responsible for the observed salinity tolerance. Several mapping populations between CPI-71284-48 and Australian cultivars already exist. While some of these have already been studied to identify traits linked to Na⁺ exclusion (Schilling et al., in preparation), the opportunity now exists to use these existing mapping populations to identify alleles and genes for shoot ion independent tolerance.

Acknowledgements

The authors would like to acknowledge funding from the Grains Research and Development Corporation, Australia (UA00118 and UA00145) and the Adelaide Barley Breeders (University of Adelaide) for advice and plant material. The Plant Accelerator[®], Australian Plant Phenomics Facility, is funded by the National Collaborative Research Infrastructure Strategy (NCRIS) of the Commonwealth of Australia. Accession YU6472 was supplied through a China Tasmanian Institute of Agriculture and Grains Research and Development Corporation collaboration and Parent 19 was sourced from the International Center for Agricultural Research in the Dry Areas (ICARDA), Lebanon.

Conflict of Interest

The authors declare no conflicts of interest.

References

- ABARES (2014) 'Agricultural commodity statistics 2014.' (Australian Government: Canberra)
- Adem, G, Roy, S, Zhou, M, Bowman, J, Shabala, S (2014) Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. *BMC Plant Biology* **14**, 113.
- Al-Tamimi, N, Brien, C, Oakey, H, Berger, B, Saade, S, Shwen Ho, Y, Schmöckel, SM, Tester, M, Negrão, S (2016) Salinity tolerance loci revealed in rice using high-throughput non-invasive phenotyping. *Nature Communications* **7**: 13342
- Berger, B, de Regt, B, Tester, M (2012) High-throughput phenotyping of plant shoots. In 'High-Throughput Phenotyping in Plants.' (Ed. J Normanly.) pp. 9 - 20. (Humana Press: New York)
- Berger, B, Parent, B, Tester, M (2010) High-throughput shoot imaging to study drought responses. *Journal of Experimental Botany* **61**, 3519-3528.
- Bhandal, IS, Malik, CP, Bourne, GH, Jeon, KW, Friedlander, M (1988) Potassium estimation, uptake, and its role in the physiology and metabolism of flowering plants. *International Review of Cytology* **110**, 205-254.
- Blaha, G, Stelzl, U, Spahn, CMT, Agrawal, RK, Frank, J, Nierhaus, KH (2000) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. In 'Methods in Enzymology.' Vol. 317 pp. 292-306. (Academic Press:
- Brien, C, Berger, B, Rabie, H, Tester, M (2013) Accounting for variation in designing greenhouse experiments with special reference to greenhouses containing plants on conveyor systems. *Plant Methods* **9**, 5.
- Butler, DG, Cullis, BR, Gilmour, AR, Gogel, BJ (2010) 'Analysis of Mixed Models for S language environments: ASReml-R reference manual.' (DPI Publications: Brisbane)
- Cassman, KG, Dobermann, A, Walters, DT, Yang, H (2003) Meeting cereal demand while protecting natural resources and improving environmental quality. *Annual Review of Environment and Resources* **28**, 315-358.
- Chen, THH, Murata, N (2011) Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. *Plant Cell & Environment* **34**, 1-20.
- Chen, Z, Newman, I, Zhou, M, Mendham, N, Zhang, G, Shabala, S (2005) Screening plants for salt tolerance by measuring K⁺ flux: a case study for barley. *Plant Cell & Environment* **28**, 1230 - 1246.
- Chen, Z, Pottosin, I, Cuin, T, Fuglsang, A, Tester, M, Jha, D, Zepeda-Jazo, I, Zhou, M, Palmgren, M, Newman, I, Shabala, S (2007) Root plasma membrane transporters controlling K⁺/Na⁺ homeostasis in salt-stressed barley. *Plant Physiology* **145**, 1714 - 1725.
- Chen, Z, Shabala, S, Mendham, N, Newman, I, Zhang, G, Zhou, M (2008) Combining Ability of Salinity Tolerance on the Basis of NaCl-Induced K Flux from Roots of Barley. *Crop Science* **48**, 1382-1388.
- Choi, W-G, Toyota, M, Kim, S-H, Hilleary, R, Gilroy, S (2014) Salt stress-induced Ca²⁺ waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proceedings of the National Academy of Sciences* **111**, 6497-6502.
- Colmer, TD, Flowers, TJ, Munns, R (2006) Use of wild relatives to improve salt tolerance in wheat. *Journal of Experimental Botany* **57**, 1059-1078.
- Colmer, TD, Munns, R, Flowers, TJ (2005) Improving salt tolerance of wheat and barley: future prospects. *Australian Journal of Experimental Agriculture* **45**, 1425-1443.
- Coombes, NE (2009) 'DiGger design search tool in R.' Available at <http://www.austatgen.org/files/software/downloads/>

526 Cuin, T, Tian, Y, Betts, S, Chalmandrier, R, Shabala, S (2009) Ionic relations and osmotic
527 adjustment in durum and bread wheat under saline conditions. *Functional Plant Biology*
528 **36**, 1110 - 1119.

529 Cuin, TA, Betts, SA, Chalmandrier, R, Shabala, S (2008) A root's ability to retain K⁺ correlates
530 with salt tolerance in wheat. *Journal of Experimental Botany* **59**, 2697-2706.

531 Eglinton JK, Coventry SJ, Mather DE, Kretschmer J, McMichael GL, Chalmers KJ (2004)
532 Advanced backcross QTL analysis in barley. *In: Proceedings of the 9th International*
533 *Barley Genetics Symposium, Brno, Czech Republic*, p 65

534 Eynard, A, Lal, R, Wiebe, K (2005) Crop response in salt affected soils. *Journal of Sustainable*
535 *Agriculture* **27**, 5-50.

536 FAOSTAT (2015) 'Food and agriculture organisation of the United Nations Statistics Division.'
537 Available at <http://faostat3.fao.org/home/E>

538 Flowers, TJ, Colmer, TD (2008) Salinity tolerance in halophytes. *New Phytologist* **179**, 945-
539 963.

540 Fricke, W, Akhiyarova, G, Veselov, D, Kudoyarova, G (2004) Rapid and tissue-specific
541 changes in ABA and in growth rate in response to salinity in barley leaves. *Journal of*
542 *Experimental Botany* **55**, 1115-1123.

543 Fricke, W, Peters, WS (2002) The biophysics of leaf growth in salt-stressed barley. A study at
544 the cell level. *Plant Physiology* **129**, 374-388.

545 Golzarian, MR, Frick, RA, Rajendran, K, Berger, B, Roy, SJ, Tester, M, Lun, DS (2011)
546 Accurate inference of cereal plant biomass using information extracted from high-
547 throughput plant images, *Plant Methods* **7**:2

548 Hairmansis, A, Berger, B, Tester, M, Roy, S (2014) Image-based phenotyping for non-
549 destructive screening of different salinity tolerance traits in rice. *Rice* **7**, 16.

550 Honsdorf, N, March, TJ, Berger, B, Tester, M, Pillen, K (2014) High-throughput phenotyping
551 to detect drought tolerance QTL in wild barley introgression lines. *PLOS One* **9**,
552 e97047.

553 Huber, AE, Bauerle, TL (2016) Long-distance plant signaling pathways in response to multiple
554 stressors: the gap in knowledge. *Journal of Experimental Botany* **67**, 2063-2079.

555 Ismagul, A, Mazonka, I, Callegari, C, Eliby, S (2014) *Agrobacterium*-mediated transformation
556 of barley (*Hordeum vulgare* L.). *In 'Crop Breeding: Methods and Protocols.'* (Eds D
557 Fleury, R Whitford.) pp. 203-211. (Springer New York: New York, NY)

558 Jacoby, RP, Che-Othman, MH, Millar, AH, Taylor, NL (2016) Analysis of the sodium
559 chloride-dependent respiratory kinetics of wheat mitochondria reveals differential
560 effects on phosphorylating and non-phosphorylating electron transport pathways. *Plant*
561 *Cell & Environment* **39**, 823-833.

562 James, R, Sv, C, Condon, A, Zawrt, A, Munns, R (2008) Genetic variation in tolerance to the
563 osmotic stress component of salinity stress in durum wheat. *Functional Plant Biology*
564 **35**, 111 - 123.

565 James, RA, Blake, C, Zwart, AB, Hare, RA, Rathjen, AJ, Munns, R (2012) Impact of ancestral
566 wheat sodium exclusion genes *Nax1* and *Nax2* on grain yield of durum wheat on saline
567 soils. *Functional Plant Biology* **39**, 609-618.

568 Kudla, J, Batistic, O, Hashimoto, K (2010) Calcium signals: The lead currency of plant
569 information processing. *The Plant Cell* **22**, 541-563.

570 Maas, EV, Hoffman, GJ (1977) Crop salt tolerance - current assessment. *Journal of Irrigation*
571 *and Drainage* **103**, 115-134.

572 Matthews, P, D., M, L., J (2015) 'Winter crop variety sowing guide 2015.' (NSW Department
573 of Primary Industries: New South Wales, Australia)

574 Mittler, R, Vanderauwera, S, Suzuki, N, Miller, G, Tognetti, VB, Vandepoele, K, Gollery, M,
575 Shulaev, V, Van Breusegem, F (2011) ROS signaling: the new wave? *Trends in Plant*
576 *Science* **16**, 300-309.

577 Munns, R (2002) Comparative physiology of salt and water stress. *Plant Cell & Environment*
578 **25**, 239-250.

579 Munns, R, James, RA, Lauchli, A (2006) Approaches to increasing the salt tolerance of wheat
580 and other cereals. *Journal of Experimental Botany* **57**, 1025-1043.

581 Munns, R, James, RA, Xu, B, Athman, A, Conn, SJ, Jordans, C, Byrt, CS, Hare, RA, Tyerman,
582 SD, Tester, M, Plett, D, Gilliam, M (2012) Wheat grain yield on saline soils is
583 improved by an ancestral Na⁺ transporter gene. *Nature Biotechnology* **30**, 360-364.

584 Munns, R, Passioura, JB, Guo, J, Chazen, O, Cramer, GR (2000) Water relations and leaf
585 expansion: importance of time scale. *Journal of Experimental Botany* **51**, 1495-1504.

586 Munns, R, Schachtman, DP, Condon, AG (1995) The significance of a two-phase growth
587 response to salinity in wheat and barley. *Australian Journal of Plant Physiology* **22**,
588 561-569.

589 Munns, R, Tester, M (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology*
590 **59**, 651-681.

591 Neilson, EH, Edwards, AM, Blomstedt, CK, Berger, B, Møller, BL, Gleadow, RM (2015)
592 Utilization of a high-throughput shoot imaging system to examine the dynamic
593 phenotypic responses of a C4 cereal crop plant to nitrogen and water deficiency over
594 time. *Journal of Experimental Botany* **66**, 1817-1832.

595 Obsa, BT, Eglinton, J, Coventry, S, March, T, Langridge, P, Fleury, D (2016) Genetic analysis
596 of developmental and adaptive traits in three doubled haploid populations of barley
597 (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **129**, 1139-1151.

598 Parent, B, Shahinnia, F, Maphosa, L, Berger, B, Rabie, H, Chalmers, K, Kovalchuk, A,
599 Langridge, P, Fleury, D (2015) Combining field performance with controlled
600 environment plant imaging to identify the genetic control of growth and transpiration
601 underlying yield response to water-deficit stress in wheat. *Journal of Experimental*
602 *Botany* **66**, 5481 - 5492.

603 Passioura, JB, Munns, R (2000) Rapid environmental changes that affect leaf water status
604 induce transient surges or pauses in leaf expansion rate. *Australian Journal of Plant*
605 *Physiology* **27**, 941-948.

606 Paynter, B, Hills, A, Gupta, S, Collins, S, Dhammu, H, Malik, R, Trainor, G (2014) '2015
607 barley variety sowing guide for Western Australia.' (Department of Agriculture and
608 Food, Government of Western Australia: Perth, Western Australia)

609 Poustini, K, Siosemardeh, A (2004) Ion distribution in wheat cultivars in response to salinity
610 stress. *Field Crops Research* **85**, 125-133.

611 Rahnama, A, Poustini, K, Tavakkol-Afshari, R, Ahmadi, A, Alizadeh, H (2011) Growth
612 properties and ion distribution in different tissues of bread wheat genotypes (*Triticum*
613 *aestivum* L.) differing in salt tolerance. *Journal of Agronomy and Crop Science* **197**, 21
614 - 30.

615 Rajendran, K, Tester, M, Roy, SJ (2009) Quantifying the three main components of salinity
616 tolerance in cereals. *Plant Cell & Environment* **32**, 237-249.

617 Rengasamy, P, Marchuk, A (2011) Cation ratio of soil structural stability (CROSS). *Soil*
618 *Research* **49**, 280-285.

619 Roy, SJ, Negrão, S, Tester, M (2014) Salt resistant crop plants. *Current Opinion in*
620 *Biotechnology* **26**, 115-124.

621 Schilling, RK, Marschner, P, Shavrukov, Y, Berger, B, Tester, M, Roy, SJ, Plett, DC (2014)
622 Expression of the Arabidopsis vacuolar H⁺-pyrophosphatase gene (*AVP1*) improves the

shoot biomass of transgenic barley and increases grain yield in a saline field. *Plant Biotechnology Journal* **12**, 378-386.

Schmöckel, SM, Garcia, AF, Berger, B, Tester, M, Webb, AAR, Roy, SJ (2015) Different NaCl-induced calcium signatures in the *Arabidopsis thaliana* ecotypes Col-0 and C24. *PLOS One* **10**, e0117564.

Shabala, S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Annals of Botany* **112**, 1209-1221.

Shavrukov, Y, Gupta, N, Miyazaki, J, Baho, M, Chalmers, K, Tester, M, Langridge, P, Collins, N (2010a) *HvNax3*—a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Functional & Integrative Genomics* **10**, 277-291.

Shavrukov, Y, Langridge, P, Tester, M, Nevo, E (2010b) Wide genetic diversity of salinity tolerance, sodium exclusion and growth in wild emmer wheat, *Triticum dicoccoides*. *Breeding Science* **60**, 426-435.

Shipley, B, Hunt, R (1996) Regression Smoothers for Estimating Parameters of Growth Analyses. *Annals of Botany* **78**, 569-576.

Sirault, X, James, R, Furbank, R (2009) A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. *Functional Plant Biology* **36**, 970 - 977.

Sutton, T, Baumann, U, Hayes, J, Collins, NC, Shi, B-J, Schnurbusch, T, Hay, A, Mayo, G, Pallotta, M, Tester, M, Langridge, P (2007) Boron-Toxicity Tolerance in Barley Arising from Efflux Transporter Amplification. *Science* **318**, 1446-1449.

Tajbakhsh, M, Zhou, MX, Chen, ZH, Mendham, NJ (2006) Physiological and cytological response of salt-tolerant and non-tolerant barley to salinity during germination and early growth. *Australian Journal of Experimental Agriculture* **46**, 555-562.

Teakle, NL, Tyerman, SD (2010) Mechanisms of Cl⁻ transport contributing to salt tolerance. *Plant Cell & Environment* **33**, 566-589.

Tester, M, Langridge, P (2010) Breeding technologies to increase crop production in a changing world. *Science* **327**, 818-822.

The R Project for Statistical Computing (2014) 'R: A language and environment for statistical computing.' Available at <http://www.r-project.org>

Thoday-Kennedy, EL, Jacobs, AK, Roy, SJ (2015) The role of the CBL–CIPK calcium signalling network in regulating ion transport in response to abiotic stress. *Plant Growth Regulation* **76**, 3-12.

Tilbrook, J, Roy, SJ (2014) Salinity tolerance. In 'Plant abiotic stress (2nd edition).' (Eds MA Jenks, PM Hasegawa.) pp. 134-178. (Wiley-Blackwell: New York)

Victoria, So (Ed. K Hollaway (2015) 'Victorian winter crop summary 2015.' (Department of Economic Development, Jobs, Transport and Resources, Victorian Government:

Wheeler, R (2015) Barley variety sowing guide 2015. In 'South Australian sowing guide 2015.' pp. 16-20. (SARDI: Adelaide, South Australia)

Widodo, Patterson, JH, Newbiggin, E, Tester, M, Bacic, A, Roessner, U (2009) Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. *Journal of Experimental Botany* **60**, 4089-4103.

Wyn Jones, RG, Brady, CJ, Spears, J (1979) Ionic and osmotic relations in plant cells. In 'Recent advances in the biochemistry of cereals.' (Eds DL Laidman, RG Wyn Jones.) pp. 63-103. (Academic Press: London)

Yeo, AR, Lee, S, Izard, P, Boursier, PJ, Flowers, TJ (1991) Short- and long-term effects of salinity on leaf growth in rice (*Oryza sativa* L.). *Journal of Experimental Botany* **42**, 881-889.

672 Zhu, GY, Kinet, JM, Lutts, S (2001) Characterization of rice (*Oryza sativa* L.) F3 populations
673 selected for salt resistance. I. Physiological behaviour during vegetative growth.
674 *Euphytica* **121**, 251-263.

675 **Tables**

676 **Table 1.** Ranking of salt tolerance (projected shoot area, ratio of pixels in salt treated:control plants) of
 677 barley plants, 17 days after treatment with 250 mM NaCl, shown in the context of the other traits
 678 measured: ion independent tolerance (ratio of the absolute growth rate calculated from days 0 to 5 after
 679 treatment), leaf health (median green:yellow pixel ratio at day 17), leaf water content (mean at day 17),
 680 leaf [Na⁺] and [K⁺] (mean, 17 days after salt treatment) and the K⁺/Na⁺ ratio. Accessions WI4304 and
 681 WI4330 are genotypes developed as part of the University of Adelaide Barley Breeding Program and
 682 have not been rated for malting quality as they are not commercially available. (Mean, n = 5-8 except
 683 for * (ion measurements of Gairdner) n = 1). Refer to Figure 5 for s.e.m values for Na⁺ and K⁺
 684 accumulation.

Genotype	Grain Use	Ion independent tolerance	Na ⁺ accumulation (mM)	K ⁺ accumulation (mM)	K ⁺ /Na ⁺	Leaf Water Content (g)	Leaf health	Salt tolerance
Skiff	Malting	0.818	197.9	173.8	0.88	0.092	0.923	0.491
Parent 19	Landrace	0.918	213.2	164.1	0.77	0.234	0.944	0.477
Fathom	Feed	0.982	193.0	171.5	0.89	0.229	0.979	0.461
Sahara 3771	Landrace	0.794	121.7	199.3	1.64	0.390	0.979	0.426
CPI 71284-48	<i>Spontaneum</i>	0.824	91.9	234.3	2.55	0.449	0.984	0.418
Schooner	Malting	0.746	318.3	199.7	0.63	0.061	0.944	0.381
Mundah	Feed	0.700	168.3	182.6	1.08	0.258	0.935	0.322
CM72	Feed	0.732	254.1	135.1	0.53	0.227	0.906	0.311
Fleet	Feed	0.682	282.3	182.8	0.65	0.128	0.881	0.278
Barque 73	Feed	0.723	302.0	171.9	0.57	0.272	0.928	0.269
Flagship	Malting	0.567	484.2	278.7	0.58	0.088	0.809	0.269
Keel	Feed	0.741	728.4	478.0	0.66	0.041	0.715	0.258
Baudin	Malting	0.635	612.7	298.3	0.49	0.063	0.804	0.245
YU6472	Feed	0.676	757.9	220.3	0.29	0.205	0.728	0.227
Vlamingh	Malting	0.645	737.5	290.4	0.39	0.100	0.635	0.223
Hindmarsh	Feed	0.611	925.9	401.7	0.43	0.015	0.641	0.220
Sloop SA	Malting	0.508	738.0	329.7	0.45	0.050	0.605	0.187
Commander	Malting	0.576	879.4	300.0	0.34	0.080	0.602	0.142
WI4304	Undetermined	0.538	912.8	393.6	0.43	0.066	0.633	0.142
Clipper	Malting	0.460	870.7	374.1	0.43	0.069	0.504	0.117
Yarra	Feed	0.564	848.2	187.5	0.22	0.127	0.579	0.116
WI4330	Undetermined	0.388	903.7	320.1	0.35	0.093	0.681	0.106
Navigator	Malting	0.376	896.0	228.9	0.26	0.114	0.642	0.101
Gairdner	Malting	0.500	1097.6*	138.4*	0.13*	0.036*	0.545	0.085

685

687 **Figure 1.** Projected shoot area (biomass) determined from the final imaging point, 17 days after (a) 0
688 mM (b) 150 mM or (c) 250 mM NaCl treatments. Salinity tolerance was determined as the ratio of the
689 projected leaf area after (d) 150 or (e) 250 mM NaCl treatment, relative to the projected shoot area of
690 control (0 mM) plants, at the final imaging time point. Fathom, Sahara 3771 and Skiff maintained a

691 higher shoot area ratio at both levels of salt treatment. This contrasted with CPI 71284-48 and Parent
692 19, which maintained a high shoot area ratio at 250 mM NaCl. Results are the predicted means and their
693 s.e.m. (n=5-8, *Gairdner n=1 at 250 mM as all other plants died).

695 **Figure 2.** Relative growth rates of each individual barley plant before and after application of (a) 0, (b)
 696 150 or (c) 250 mM NaCl. They are calculated from the smoothed growth curves. Salt application took
 697 place at day 0. The red line is the average relative growth rate for all plants within the experiment.

Figure 3. The ratio of the relative growth rates through time for each of the 24 barley lines grown with either 150 mM NaCl or 250 mM NaCl treatments. The ratio was determined as the relative growth rate under salt stress ÷ relative growth rate in 0 mM NaCl. The rates between the dotted vertical lines (days 0 to 5) are used to calculate the shoot ion independent tolerance. Results are the predicted means and their s.e.m. (n = 5-8, *Gairdner n=1 at 250 mM as all other plants died).

Figure 4. The shoot ion independent tolerance of barley seedlings over a 5 day period immediately following treatment with (a) 150 mM or (b) 250 mM NaCl. A positive relationship can be observed between shoot ion independent tolerance and overall plant salinity tolerance at (c) 150 mM and (d) 250 mM NaCl. The shoot ion independent tolerance is expressed as a ratio of relative shoot growth rates at either 150 mM or 250 mM NaCl ÷ 0 mM NaCl over the first 5 days of salt stress. Salt tolerance was determined by projected shoot area salt ÷ projected shoot area control 17 days after treatment with 250 mM NaCl. Differences in tolerance was seen between lines at both treatment levels (n = 5-8, *Gairdner n=1 at 250 mM as all other plants died, results are the mean ± s.e.m.). Pearson correlation analysis results including r, R² and P values are shown.

Figure 5. Accumulation of Na^+ (a, c and e) and K^+ (b, d and f) in leaf blades after 17 days of growth in 0, 150 or 250 mM NaCl ($n = 5-8$, s.e.m. *Gairdner $n = 1$ at 250 mM as all other plants died).

Figure 6. Mean leaf $[\text{Na}^+]$ vs (a) mean projected shoot area or (b) salt tolerance (projected shoot area salt $\div$ projected shoot area control) 17 days after treatment with 150 mM NaCl. Mean leaf $[\text{Na}^+]$ vs (c) mean projected shoot area or (d) salt tolerance (projected shoot area salt $\div$ projected shoot area control) 17 days after treatment with 250 mM NaCl. (e) Leaf health index (number of green pixels $\div$ total plant pixels) for all 24 lines after 17 days at 250 mM NaCl. (f) Leaf health index (number of green pixels $\div$ total plant pixels) vs salt tolerance (projected shoot area salt $\div$ projected shoot area control) 17 days

746 after treatment with 250 mM NaCl (Mean values shown, n = 5-8, *Gairdner n = 1 at 250 mM). Pearson
747 correlation analysis results including r, R^2 and P values are shown. n.s. = non-significant.

Supplementary Files

Supplementary Table 1. Ranking of the shoot ion independent salt tolerance of barley under 150 mM NaCl treatment, shown in the context of the other traits measured: ion independent tolerance (ratio of the absolute growth rate calculated from days 0 to 5 after treatment), leaf water content (mean at 17 days), leaf health (median green:yellow pixel ratio at day 17), leaf [Na⁺] and [K⁺] (mean, 17 days after salt treatment) and the K⁺/Na⁺ ratio. Accessions WI4304 and WI4330 are genotypes developed as part of the University of Adelaide Barley Breeding Program and have not been rated for malting quality as they are not commercially available. (Mean, n = 5-8).

Genotype	Grain Use	Ion independent tolerance	Na ⁺ accumulation (mM)	K ⁺ accumulation (mM)	K ⁺ /Na ⁺	Leaf water content (g)	Leaf health	Salt tolerance
Fathom	Feed	1.027	93.7	253.8	2.71	0.055	0.994	0.694
CM72	Feed	1.000	169.5	172.5	1.02	0.322	0.993	0.657
Skiff	Malting	0.866	113.4	225.3	1.99	0.092	0.991	0.638
Mundah	Feed	0.869	99.2	240.4	2.42	0.436	0.992	0.636
Sahara 3771	Landrace	0.894	98.9	237.1	2.40	0.076	0.990	0.623
Keel	Feed	0.810	128.5	212.4	1.65	0.040	0.984	0.615
Schooner	Malting	0.841	139.8	186.0	1.33	0.055	0.992	0.610
YU6472	Feed	0.859	99.7	255.0	2.56	0.181	0.992	0.601
Flagship	Malting	0.933	115.4	217.6	1.88	0.086	0.991	0.598
Hindmarsh	Feed	0.874	126.6	197.8	1.56	0.049	0.987	0.582
Baudin	Malting	0.886	107.0	215.3	2.01	0.050	0.990	0.569
Vlamingh	Malting	0.825	154.6	159.9	1.03	0.089	0.982	0.568
Commander	Malting	0.881	163.2	186.3	1.14	0.068	0.988	0.562
Barque73	Feed	0.832	106.7	242.0	2.27	0.323	0.991	0.552
Parent19	Landrace	0.698	176.7	323.4	1.83	0.138	0.989	0.540
CPI 71284-48	<i>Spontaneum</i>	0.855	118.0	225.6	1.91	0.507	0.994	0.520
Gairdner	Malting	0.873	103.8	213.2	2.05	0.384	0.989	0.518
WI4330	Undetermined	0.954	195.6	169.1	0.86	0.111	0.981	0.492
Clipper	Malting	0.806	193.3	200.8	1.04	0.061	0.978	0.471
Fleet	Feed	0.931	100.5	272.2	2.71	0.135	0.992	0.469
Yarra	Feed	0.863	144.6	183.8	1.27	0.141	0.987	0.462
Navigator	Malting	0.795	173.0	175.2	1.01	0.096	0.984	0.444
WI4304	Undetermined	0.796	116.1	189.0	1.63	0.019	0.965	0.439
Sloop SA	Malting	0.722	360.7	303.3	0.84	0.043	0.850	0.323

Supplementary Figure 1. Performance of barley lines when group by their malting classification for (a) salinity tolerance (size of plant in 250 mM NaCl ÷ size of plant in 0 mM NaCl), (b) shoot ion independent tolerance and (c) accumulation of Na⁺ in the leaf. Results which are significantly different $P \leq 0.05$ (One-way ANOVA) are indicated with different letters. Recent landrace derived lines were included in the landrace classification. Varieties were classified according to malting or feed characteristics. However, most malting varieties can also be used as feed barley. The landrace derived lines are significantly different to the malting and feed varieties for all three traits.

Supplementary Figure 2. Growth of barley over a period of 21 days with NaCl treatment of 0 (blue), 150 (dark red) or 250 (light red) mM applied at day 0. The period between 0 and 5 days after treatment (indicated with the vertical broken lines) was used to calculate the shoot ion independent tolerance, before ions were able to accumulate to high concentrations in the shoot and significantly affect shoot

769 function. Values plotted are the mean and s.e.m. of the mean ($n = 5-8$ *Gairdner $n=1$ at 250 mM as all
770 other plants died).

Supplementary Figure 3. Relationship between mean relative shoot growth rate for each cultivar at 0 mM NaCl and the cultivar's ion independent tolerance at 250 mM NaCl.